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Manduca sexta Moricin Promoter Elements can Increase Promoter Activities of *Drosophila melanogaster* Antimicrobial Peptide Genes

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Abstract

Insects produce a variety of antimicrobial peptides (AMPs). Induction of insect AMP genes is regulated by the Toll and IMD (immune deficiency) pathways via NF-κB and GATA factors. Little is known about species-specific regulation of AMP genes. In this report, we showed that activities of most *Manduca sexta* and *Drosophila melanogaster* AMP gene promoters were regulated in a species-specific manner in *Drosophila* (Dipteran) S2 cells and *Spodoptera frugiperda* (Lepidopteran) Sf9 cells. A κB-GATA element (22bp) from *M. sexta moricin* (*MsMoricin*) promoter could significantly increase activities of *Drosophila* AMP gene promoters in S2 cells, and an *MsMoricin* promoter activating element (MPAE) (140bp) could increase activity of *drosomycin* promoter specifically in Sf9 cells. However, κB and GATA factors alone were not sufficient for *MsMoricin* gene activation, suggesting that other co-regulators may be required to fully activate AMP genes. Our results suggest that induction of insect AMP genes may require a transcription complex composed of common nuclear factors (such as NF-κB and GATA factors) and species-related co-regulators, and it is the co-regulators that may confer species-specific regulation of AMP genes. In addition, we showed that activity of *Drosophila drosomycin* promoter could be activated cooperatively by the inserted exogenous κB-GATA element and the endogenous κB element. These findings revealed an approach of engineering AMP genes with enhanced activities, which may lead to broad applications.

Keywords

NF-κB; GATA; *Drosophila melanogaster*; *Manduca sexta*; Antimicrobial peptide; Promoter

1. Introduction

The innate immune system is conserved from insects to humans (Ganesan et al., 2011; Mogensen, 2009). Insect innate immune system relies on various germ line encoded pattern recognition receptors (PRRs) to sense pathogen-associated molecular patterns (PAMPs) and induce cellular and humoral responses (Charroux et al., 2009; Charroux and Royet, 2010;

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Kanost et al., 2004; Lemaitre and Hoffmann, 2007; Marmaras and Lampropoulou, 2009). An important aspect of insect humoral responses is induced production of a variety of antimicrobial peptides (AMPs) (Diamond et al., 2009). Most AMPs are small cationic peptides with activities against microorganisms and parasites (Brogden, 2005; Imler and Bulet, 2005; Kokoza et al., 2010). In *Drosophila melanogaster*, induced production of AMPs is regulated by the Toll and IMD (immune deficiency) pathways (Lemaitre et al., 1995; 1996; DeGregorio et al., 2002). The Toll pathway mediates recognition of fungi and Gram-positive bacteria (Ashok, 2009), while the IMD pathway senses infection by most Gram-negative bacteria (Kaneko and Silverman, 2005). The Toll pathway triggers activation of NF-κB factors Dorsal and Dif, while the IMD pathway leads to activation of Relish (Engström et al., 1993; Ganesan et al., 2011; Gross et al., 1996; Hetru and Hoffmann, 2009; Ip et al., 1993; Stöven et al., 2003).

GATA factors are a family of zinc finger containing transcription factors, which recognize the (A/T)GATA(A/G) consensus sequence, and they are involved in regulation of gene expression and differentiation (Patient and McGhee, 2002). GATA factors have been identified in vertebrates, *D. melanogaster*, *Caenorhabditis elegans*, and plants (Patient and McGhee, 2002; Reyes et al., 2004). Vertebrate GATA-1 interacts with many other regulatory partners, such as Friend of GATA (FOG), p300/CBP, PU.1 and c-myb (Lowry and Mackay, 2006). GATA factors interact with different factors to regulate gene transcription (Dai et al., 2002; Eisbacher et al., 2003; Gordon et al., 1997; Zhang et al., 2007). In *D. melanogaster*, Serpent (dGATAb) and dGATAe regulate induced gene expression in fat body and midgut, respectively (Petersen et al., 1999; Senger et al., 2006). GATA factors are also required for immunity in *C. elegans* and the silkworm *Bombyx mori* (Cheng et al., 2006; Kerry et al., 2006). Adjacent κB and GATA sites have been identified in many insect immune gene promoters and both sites are required for gene induction (Harshman and James, 1998; Kadalayil et al., 1997; Senger et al., 2004; Tingvall et al., 2001). Human GATA-3 and/or GATA-2 interact with NF-κB to trigger GlcNac6ST-1 transcription (Chen et al., 2008). In addition, κB and GATA sites are both required for induced expression of *Drosophila cecropin* A (Kadalayil et al., 1997). Therefore, κB-GATA synergy seems to be a common mechanism for immune gene regulation (Senger et al., 2004). However, little is known about synergistic effect of κB and GATA factors in insects.

In *D. melanogaster*, seven groups of AMPs have been identified, some AMP genes (*drosomycin*, *diptericin*, *metchnikowin*) have been identified only in *Drosophila*, others (*attacin*, *cecropin*, *drosocin*, *defensin*) are also found in other insect species (Imler and Bulet, 2005; Levashina et al., 1998). Drosomycin is an anti-fungal peptide isolated from immune challenged *D. melanogaster* (Fehlbaum et al., 1994; Tian et al., 2008). Expression of *drosomycin* is synergistically regulated by the Toll and IMD pathways (Tanji et al., 2007; Tanji et al., 2010). *Diptericin* is another species-related AMP gene first identified in *Phormia terranovae* and later in *D. melanogaster* (Dimarcq et al., 1988; Wicker et al., 1990). Different groups of AMP genes have also been identified in lepidopteran insects, such as *B. mori* and *Manduca sexta* (Kanost et al., 2004). *Moricin*, *gloverin* and *lebocin* genes have been identified only in lepidopteran insects (Axen et al., 1997; Chowdhury et al., 1995; Hara and Yamakawa, 1995; Kanost et al., 2004). Moricin was originally isolated from the hemolymph of *B. mori* and showed antibacterial activity against several Gram-negative and Gram-positive bacteria (Hara and Yamakawa, 1995, 1996). The N-terminal region of *B. mori* Moricin adopts an amphipathic alpha-helix structure that may increase permeability of the cytoplasmic membrane (Hemmi et al., 2002). Moricin analogues have been identified in other lepidopteran species, including *M. sexta*, *Galleria mellonella*, and *Spodoptera litura* (Brown et al., 2008; Oizumi et al., 2005; Zhu et al., 2003).

Our previous research reveals that lipopolysaccharide (LPS) and lipoteichoic acid (LTA) can induce AMP gene expression in *M. sexta* larvae (Rao and Yu, 2010). In *D.* melanogaster, peptidoglycan can activation AMP genes (Werner et al., 2000; 2003), but ultrapure LPS molecules do not induce AMP expression in adult flies (Kaneko et al., 2004), indicating that there may be important differences between dipteran and lepidopteran species regarding regulation of AMP genes. It is not known whether expression of AMP genes is regulated in a species-specific manner, and whether different co-regulators are involved in regulating AMP gene expression in lepidopteran and dipteran insects. In this study, we cloned promoters for *M. sexta moricin* (*MsMoricin*), *cecropin* and lysozyme genes and compared activities of the three *M. sexta* (Lepidopteran) and seven *D. melanogaster* (Dipteran) AMP gene promoters in *D. melanogaster* S2 cells and *Spodoptera frugiperda* (Lepidopteran) Sf9 cells. We found that most AMP gene promoters were regulated in a species-specific manner in the two cell lines in that *D. melanogaster* AMP gene promoters had no or low activity in Sf9 cells and *M. sexta* AMP gene promoters had no or low activity in S2 cells. We then showed that κB and GATA factors alone were not sufficient to activate *MsMoricin* promoter, and a κB-GATA element (22bp) from the *MsMoricin* promoter could significantly increase activities of *D. melanogaster* AMP gene promoters when inserted into the promoters. We also showed that the κB-GATA element and the endogenous κB site2 of *drosomycin* promoter were all required to cooperatively enhance *drosomycin* promoter activity. More importantly, we identified an activating element, designated as *MsMoricin* promoter activating element (MPAE) (140bp), which could increase activity of *drosomycin* promoter specifically in Sf9 cells, thus MPAE may contain co-regulator binding sites for nuclear factors specifically expressed in lepidopteran species. Our results suggest that common factors such as NF-κB and GATA factors are functional in both dipteran and lepidopteran insects, while co-regulators may confer species-specific regulation of AMP genes.

2. Material and methods

2.1 Insects, bacterial peptidoglycan (PG) and insect cell lines

M. sexta eggs were kindly provided by Professor Michael Kanost, Department of Biochemistry at Kansas State University. Larvae were reared on an artificial diet at 25°C (Dunn and Drake, 1983), and the 5th instar larvae were used for hemocytes collection. Ultrapure peptidoglycan from *E. coli* strain K12 (Cat#: tlrl-pgnek) was purchased from InvivoGen (San Diego, California, USA) and used for activation experiments. *Drosophila melanogaster* S2 cells were purchased from American Type Culture Collection (ATCC). *Spodoptera frugiperda* Sf9 cells were purchased from Invitrogen Corporation, USA.

2.2 Genomic DNA extraction and genome walking

M. sexta genomic DNA was extracted from hemocytes collected from the 5th instar larvae with PureLink™ Genomic DNA Kit (Invitrogen, USA). *D. melanogaster* genomic DNA was extracted from S2 cells. Genome walking was performed to clone *MsMoricin* and *MsCecropin* promoters with GenomeWalker Universal Kit (Clontech, USA) following instructions of the manufacturer. Briefly, 2.5 μg *M. sexta* genomic DNA was digested with *Dra* I, *EcoR* V, *Pvu* II or *Stu* I, respectively. Digested fragments were purified and ligated to a synthetic adaptor GWAdaptor. Adaptor primers (GW-AP1 and GW-AP2) and gene specific primers (MsMoricinGSP1-4, MsCecropinGSP1 and 2) (Table S1) were used for PCR reactions.

2.3 RNA extraction and 5′ RACE

M. sexta hemocytes were collected from the 5th instar larvae at 6 h after *E. coli* XL1-blue injection and total RNA was prepared from hemocytes with TRI reagent (Sigma Aldrich,

USA). cDNA was prepared with ImProm-II reverse transcriptase (Promega, USA). 5′ RACE was performed to determine transcription start site of *M. sexta moricin* promoter with SMARTer™ RACE cDNA amplification kit (Clontech, USA).

2.4 Sequence analysis

Transcription factor binding sites were predicted with Alibaba2.1 (http://www.gene-regulation.com/). Other sequences were analyzed with DNAMAN (Lynnon Corporation, Quebec, Canada).

2.5 Construction of luciferase reporter plasmids

For luciferase reporter plasmids, promoters from antimicrobial peptide (AMP) genes of *M. sexta* and *D. melanogaster* were cloned by genome walking or PCR using genomic DNAs as templates. PCR was performed with Taq DNA polymerase using gene specific primers listed in Table S1. For *MsMoricin*, *Ms*Lysozyme and *D. melanogaster* AMP genes reporters, PCR products were digested and ligated to the *Kpn* I/*Bgl* II sites of pGL3Basic vector (Promega, USA). For *MsCecropin* reporter, PCR product was digested and ligated to the *Xho* I and *Hind* III sites of pGL3Basic vector. *MsMoricin*, *MsCecropin*, *Ms*Lysozyme, *diptericin*, *DmAttacin* A, *DmDefensin*, *drosomycin*, *DmCecropin* A1, *drosocin* and *metchnikowin* luciferase reporters contained 1456bp, 877bp, 1241bp, 980bp, 977bp, 1651bp, 812bp, 670bp, 660bp, and 1560bp of 5′ upstream sequences, respectively. In these luciferase reporters, +1 indicates the translation start site (ATG) in *MsCecropin*, while +1 indicates transcription initiation sites for *MsMoricin*, *drosomycin*, *diptericin* and *DmAttacin* A genes. Deletion and mutation reporters were constructed by overlapping PCR. The first round of overlapping PCR was performed to amplify the 5′ and 3′ end DNA fragments individually with overlapping regions, and the second round of overlapping was done by mixing fragments amplified from the first round PCR as templates with the 5′ and 3′ primers. *MsMoricin* κB5 (GTAAAGTCCC) was mutated to *T*TA*G*AGT*TAT*, and GATA-1 (TCGTTATCTG) was mutated to TCG*CGTATC*G. *Drosomycin* κB site-1 (GGGTTTAACC) was mutated to *ATT*TTTAACC, κB site-2 (AGTAGTTCCC) was mutated to AGTAGTT*AAT*, and a predicted κB site-4 (GGACAGTCCA) was mutated to *T*GA*G*AGT*TAT*. *MsMoricin* κB-GATA element (GTAAAGTCCCTATCGTTATCTG) was mutated to mutκB-GATA (with a mutated κB site) (*T*TA*G*AGT*TAT*TATCGTTATCTG), κB-mutGATA (with a mutated GATA site) (GTAAAGTCCCTATCG*AA*A*AAC*G), or mutκB-mutGATA (with both mutated κB and GATA sites) (*T*TA*G*AGT*TAT*TATCG*AA*A*AAC*G). To make insertion constructs, κB-GATA, mutκB-GATA, κB-mutGATA, mutκB-mutGATA, GATA (TATCGTTATCTGAGAG), MPAE (−242 to −57), MPAE-κB (−242 to −47), and MPAE-κB-GATA (−242 to −35) were inserted into different promoters at positions indicated in figures, respectively. Plasmids for transfection were prepared with PureYield™ Plasmid Miniprep System (Promega, USA). Sequences of the promoters are given in Texts S1–S9, where known or predicted NF-κB elements and GATA elements are noted by single and double underlines, respectively. Transcriptional/Translational start sites are shown in boxes.

2.6 Insect cell culture and transfection

Drosophila Schneider 2 (S2) cells and *S. frugiperda* Sf9 cells were maintained at 27°C in TNM-FH (HyClone, USA) supplemented with 10% fetal bovine serum, 1×L-Glutamine, 50 IU/mL penicillin and 0.05 mg/mL streptomycin (Hyclone, USA). For DNA transfection, 10^4 cells were plated in each well of a 96-well plate and transfected with 150 ng reporter plasmid and 15 ng pRL-TK renilla luciferase plasmid as an internal control (Promega, USA) for 12 h. Then fresh medium containing 10 μg/mL PG-K12 was used to stimulate cells for 48 h before measuring the luciferase activities. Even though 20-hydroxyecdysone-treated cells are more sensitive to immune challenges (Dimarcq et al., 1997; Silverman et al., 2000),

we did not use the hormone to treat cells in our experiments because our purpose was to observe increase in promoter activities activated by PG-K12. Each transfection was performed in three wells independently and transfection experiments were repeated three times. Firefly and renilla luciferase activities were measured using Dual-luciferase Reporter Assay system (Promega, USA). Briefly, S2 cells or Sf9 cells in 96-well plates were washed once with sterile PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), and then lysed with 30 μL 1× lysis buffer (Promega, USA) at room temperature for 15 min with shaking. The cell lysate (30 μL) was transferred to a tube containing 100 μL luciferase assay reagent II and mixed well, then reading was recorded immediately, 100 μL stop & glo reagent was added to quench the first reaction and the control renilla luciferase activity was measured using a Liquid Scintillation Counter (Cat #: 425-034, HIDEX, Turku, Finland).

2.7 Data analysis

Figures were made with the GraphPad Prism software with one representative set of data. Significance of difference was determined by an unpaired t-test or by one way ANOVA followed by a Tukey's multiple comparison test using the same software (GraphPad, San Diego, CA).

3. Results

3.1 Species-specific regulation of AMP gene promoters in S2 and Sf9 cells

We cloned a 1.4-kb 5′-regulatory fragment of *M. sexta moricin* (*MsMoricin*) gene (GenBank accession number: JF309316.1) and constructed a luciferase reporter. Initial activity assay in *D. melanogaster* S2 cells (a dipteran cell line) and *S. frugiperda* Sf9 cells (a lepidopteran cell line) showed that *MsMoricin* promoter did not have activity in S2 cells, but had relatively high activity in Sf9 cells after peptidoglycan (PG) stimulation (Figure 1). This result suggests that insect AMP genes may be regulated in a species-specific manner. We used PG-K12 (from *E. coli* K12) to directly stimulate S2 and Sf9 cells since we did not overexpress any Rel/NF-κB proteins in these cells, and PG-K12 can bind to cell surface peptidoglycan-recognition proteins (PGRPs) to activate the IMD pathway in *Drosophila* (Kaneko et al., 2004). Thus, the activity observed is due to activation of promoters by endogenous transcription factors in S2 or Sf9 cells. Activation of the Toll pathway by Lys-type peptidoglycan from Gram-positive bacteria not only requires PGRPs but also involves activation of proteinases and Spätzle (ligand for the Toll receptor) (Ganesan et al., 2011), thus we did not use Gram-positive peptidoglycan in our study. All the following experiments in S2 and Sf9 cells were stimulated with PG-K12.

To further test whether insect AMP gene promoters indeed are regulated in a species-specific manner, we cloned two more *M. sexta* AMP promoters (*MsCecropin* and *Ms*Lysozyme) and seven *D. melanogaster* AMP promoters (*drosomycin* (*Drs*), *diptericin* (*Dpt*), *drosocin*, *metchnikowin*, *DmCecropin* A1, *DmAttacin* A, and *DmDefensin*), and constructed these promoters as luciferase reporters. Results from dual luciferase assays showed that *MsMoricin* and *MsCecropin* promoters were active only in Sf9 cells; *drosomycin*, *DmDefensin*, *DmCecropin* A1, *metchnikowin* and *drosocin* promoters were either active only in S2 cells or showed significantly higher activities in S2 cells than in Sf9 cells (Figure 1). *DmAttacin* A and *diptericin* promoters had lower activities in both S2 and Sf9 cells, and *Ms*Lysozyme promoter showed similarly high activities in both cell lines (Figure 1). These results suggest that some AMP gene promoters indeed are regulated in a species-specific manner.

3.2 Identification of an active κB-GATA element in MsMoricin promoter

In order to identify promoter regulatory elements, we first studied regulation of *MsMoricin* gene. Analysis of *MsMoricin* promoter sequence showed that there are five predicted κB sites and eight GATA sites with one GATA-1 site only 2bp downstream of the κB5 (Figure 2A). Deletion of the κB1, 2, 3 or 4 did not have an effect on *MsMoricin* promoter activity; however, deletion of the κB5 almost completely abolished promoter activity in Sf9 cells (Figure 2B). Deletion or mutation of either the κB5 or the adjacent GATA-1 site significantly decreased *MsMoricin* promoter activity in Sf9 cells (Figure 2C), indicating that the κB5 and GATA sites function together as an active element required for activation of *MsMoricin* promoter.

3.3 Identification of an activating element upstream of the MsMoricin κB-GATA element

To further identify active elements in *MsMoricin* promoter, we made several deletion constructs and found that a short promoter of about 240 bp (*Mor0.24*) was fully active as 1.4kb *MsMoricin* promoter (Figure 3). Further deletions to the 240-bp promoter caused gradually loss in promoter activities. Noticeably, *Mor0.1* (0.1kb), a construct with a complete κB5-GATA element, was inactive. The region between *Mor0.24* and *Mor0.1* was obviously critical to promoter activity as *Mor0.24* (0.24kb) had similarly high activity as *MsMoricin* promoter (1.4kb) did, but *Mor0.1* (0.1kb) almost had no activity (Figure 3). These results indicated that the κB-GATA element was necessary but not sufficient to activate *MsMoricin* promoter, and the region between -240bp and −100bp in the promoter (designated as *Moricin* Promoter Activating Element, MPAE) may contain important co-regulator binding sites, and these co-regulators are also required to fully activate *moricin* gene.

3.4 The κB-GATA element of MsMoricin can enhance promoter activities of AMP genes

We showed that the κB-GATA element was essential but not sufficient to activate *MsMoricin* promoter (Figures 2 and 3). To test whether the κB-GATA element (22bp) from *MsMoricin* promoter can increase promoter activities of *M. sexta* and *D. melanogaster* AMP genes in S2 and/or Sf9 cells, the κB-GATA element was inserted into *MsCecropin*, *diptericin* and *DmAttacin* A promoters. *Diptericin* and *DmAttacin* A promoters were selected for the experiments since they had low activities in S2 cells (Figure 1). *MsCecropin* promoter contains six predicted GATA sites (Figure 5A). *Diptericin* and *DmAttacin* A promoters both contain a GATA site adjacent to a κB site (Senger et al., 2004) (Figures 4A and 5A), and the two κB sites in *diptericin* promoter have identical sequences (Kappler et al., 1993). When an *MsMoricin* κB-GATA element was inserted 40bp upstream of the endogenous GATA site (30bp downstream of the κB1) in *diptericin* promoter, a significantly increase in promoter activity was observed in both S2 and Sf9 cells, but insertion of mutant κB-GATA elements (either with a mutated κB or GATA site, or both) did not significantly change promoter activity (Figure 4B and C). In addition, insertion of a κB-GATA element increased *diptericin* promoter activity to a significantly higher level in S2 cells (~24-fold of *diptericin* promoter) than in Sf9 cells (~15-fold) (Figure 4B and C). Similarly, insertion of a κB-GATA element into *DmAttacin* A and *MsCecropin* promoters also significantly increased activities of the two promoters in S2 cells (Figure 5B), but did not increase promoter activities in Sf9 cells (Figure 5C). Together, these results suggest that the *MsMoricin* κB-GATA element can increase AMP promoter activity, and this element may not be species-related.

3.5 The κB-GATA element and the κB site2 cooperatively increase drosomycin promoter activity

D. melanogaster drosomycin promoter contains three κB sites, the κB site1 and site2 are mediated by the Toll and IMD pathways, respectively, and the two κB sites have synergic effects on *drosomycin* activation (Tanji et al., 2007; Tanji et al., 2010), but the κB site3 is not essential for promoter activity (Tanji et al., 2007). *Drosomycin* promoter also contains four GATA binding sites (Senger et al., 2004). Analysis of *drosomycin* promoter sequence showed another predicted κB site in between the κB site1 and site2, which we named the κB site4 (Figure 6A). In the following experiments, we focused our experiments on *drosomycin* promoter since its two κB sites are regulated differently. To test whether the predicted κB site4 is essential for *drosomycin* promoter, the κB site4 was deleted or mutated, and the results showed that deletion and mutation of the κB site4 did not decrease *drosomycin* promoter activity in S2 and Sf9 cells (Figure S1, panel A), indicating that the predicted κB site4 is not required for *drosomycin* promoter. We then replaced the κB site4 (10bp) with an *MsMoricin* κB-GATA element (22bp). Replacement of the κB site4 with a κB-GATA element significantly increased promoter activity in S2 cells (~21 folds, indicated by $4rep^{(\kappa B\text{-}GATA)}$-*Drs*), but similar replacement with mutant κB-GATA elements (with a mutated κB or GATA site or both, indicated by $4rep^{(mut\kappa B\text{-}GATA)}$-*Drs*, $4rep^{(\kappa B\text{-}mutGATA)}$-*Drs*, or $4rep^{(mut\kappa B\text{-}mutGATA)}$-*Drs*) did not significantly change activity of these promoters compared to *drosomycin* promoter (Figure 6B). These results demonstrated that an additional κB-GATA element can increase *drosomycin* promoter activity, and both the κB and GATA sites are required to increase promoter activity.

To test whether increase in *drosomycin* promoter activity by the *MsMoricin* κB-GATA element requires the endogenous κB site1 or site2, the κB site1 or site2 in the κB-GATA replaced promoter was mutated (indicated by $4rep^{(\kappa B\text{-}GATA)}$-1mut-*Drs* or $4rep^{(\kappa B\text{-}GATA)}$-2mut-*Drs*). Mutation of the site1 did not impair the κB-GATA element to increase promoter activity; however, mutation of the site2 completely abolished the ability of the κB-GATA element to enhance promoter activity in S2 cells (Figure 6B). These results suggest that the κB-GATA element and the endogenous κB site2 act cooperatively to increase *drosomycin* promoter activity. In Sf9 cells, all the site4 replacement and mutation constructs showed similar low activities as *drosomycin* promoter did (Figure S1, panel B), suggesting that the κB-GATA element cannot increase *drosomycin* promoter activity in Sf9 cells.

3.6 MPAE may contain lepidoptera-related co-regulator binding sites

We also mutated the κB site1 and site2 in *drosomycin* promoter and found that mutation of the κB site1 did not cause a loss of promoter activity in S2 cells, and mutation of the κB site2 significantly decreased promoter activity in S2 cells, but both mutations did not have an effect on promoter activity in Sf9 cells (Figure 7). These results in S2 cells were consistent with those reported previously that the κB site1 and site2 are activated by the Toll and IMD pathways, respectively (Tanji et al., 2007; Tanji et al., 2010), as PG-K12 (peptidoglycan from *E. coli* strain K12) that can activate the IMD pathway in *Drosophila* was used to stimulate S2 cells in our experiments. To test whether the MPAE (140bp), κB, GATA, or the whole MPAE-κB-GATA element (205bp, from −240 to −35bp) from *MsMoricin* promoter can increase *drosomycin* promoter activity in S2 cells and/or Sf9 cells in particular, an MPAE was inserted prior to the κB site1 or site2, or the κB site1 or site2 was replaced with an MPAE, MPAE-κB or MPAE-κB-GATA. Our results showed that insertion of an MPAE before the κB site1 did not significantly change promoter activity in S2 cells, but did significantly increase promoter activity in Sf9 cells (1.8-fold of *drosomycin* promoter) (Figure 7A and B). Replacement of the κB site1 with an MPAE increased promoter activity significantly in both S2 cells (3.5-fold) and Sf9 cells (1.7-fold) (Figure 7A

and B). Replacement of the κB site1 with an MPAE-κB did not further increase promoter activity compared to the MPAE replacement. However, replacement of the κB site1 with a whole MPAE-κB-GATA element increased promoter activity to a significantly higher level in both S2 cells (11.6-fold) and Sf9 cells (3.8-fold) (Figure 7A and B). Since the κB site 1 is not activated by PG and *drosomycin* promoter did not have activity in Sf9 cells (Figure 1), these results suggest that MPAE could specifically increase *drosomycin* promoter activity in Sf9 cells.

Drosomycin κB site2 is activated by Gram-negative peptidoglycan via the IMD pathway (Tanji et al., 2007; Tanji et al., 2010), thus mutation of the κB site2 significantly decreased promoter activity in S2 cells (0.2-fold of *drosomycin* promoter) (Figure 7C). Insertion of an MPAE prior to the κB site2 and replacement of the κB site2 with an MPAE and MPAE-κB significantly decreased promoter activity in S2 cells (0.6-, 0.25- and 0.3-fold of *drosomycin* promoter, respectively) (Figure 7C), suggesting that an MPAE or MPAE-κB could not replace the κB site2 to activate *drosomycin* in S2 cells. But insertion of an MPAE before the κB site2 significantly increased promoter activity in Sf9 cells (5.6-fold), while replacement of the κB site 2 with an MPAE and an MPAE-κB did not have an effect on promoter activity in Sf9 cells (Figure 7D). However, replacement of the κB site2 with a whole MPAE-κB-GATA element increased promoter activity to a significantly higher level in both S2 cells (1.8-fold) and Sf9 cells in particular (14.3-fold) (Figure 7C and D). Since the κB-GATA element could increase *drosomycin* promoter activity in S2 cells but not in Sf9 cells (Figures 6B and S1), the κB site2 but not the κB site1 is required for peptidoglycan stimulation (Tanji et al., 2007; Tanji et al., 2010), these results indicated that MPAE may contain some binding sites for nuclear factors expressed specifically in Sf9 cells (a lepidopteran cell line), which can increase *drosomycin* promoter activity in Sf9 cells.

To further confirm that the MPAE can increase *drosomycin* promoter activity in Sf9 cells, we replaced the predicted κB site4 (10bp), a non-essential site, with an *MsMoricin* κB-GATA or an MPAE-κB-GATA (Figure 8A and B). Replacement of the κB site4 with a κB-GATA significantly increased promoter activity in S2 cells (Figure 8A), but did not have an effect on promoter activity in Sf9 cells (Figure 8B). Replacement of the site4 with an MPAE-κB-GATA though also significantly increased promoter activity in S2 cells compared to the control *drosomycin* promoter, but decreased promoter activity compared to the κB-GATA replacement (Figure 8A), suggesting that an MPAE did not further increase (actually decreased) promoter activity in S2 cells. However, the MPAE-κB-GATA replacement significantly increased promoter activity in Sf9 cells (Figure 8B), indicating that MPAE indeed contains lepidoptera-related co-regulator binding sites that can increase *drosomycin* promoter activity in Sf9 cells. We also inserted an *MsMoricin* GATA site just after the κB site1 and the site2 of *drosomycin* promoter and our results showed that a GATA insertion after the κB site1 significantly increased promoter activity in S2 cells (5.8-fold), but did not increase promoter activity in Sf9 cells, and a GATA insertion after the κB site2 did not have an effect on promoter activity in both S2 and Sf9 cells (Figures 8C and S1).

4 Discussion

4.1 Species-specific regulation of AMP genes

Most AMP promoters investigated in this study showed species-specific regulation (Figure 1), suggesting that certain components in the transcription complex may account for the species-specific regulation. Since the κB and GATA transcription factors bind to similar consensus sequences across different species, *M. sexta* κB-GATA element may not be species-related and therefore is functional in *D. melanogaster* S2 cells. We hypothesize that transcription of insect AMP genes may involve formation of a transcription complex composed of both common factors (NF-κB and GATA) and species-related co-regulators,

and it is the co-regulator that confers species-specific regulation. Indeed, some AMPs are species-specific. For example, *moricin*, *gloverin* and *lebocin* genes have been identified only in lepidopteran insects so far (Axen et al., 1997; Chowdhury et al., 1995; Hara and Yamakawa, 1995). *D. melanogaster* deformed epidermal autoregulatory factor-1 (DEAF-1) has been identified as a new factor that contributes to induced expression of *metchnikowin* and *drosomycin* (Gross and McGinnis, 1996; Reed et al., 2008), two species-specific AMP genes in *Drosophila*. In addition, Dorsal interacting proteins have been identified (Li et al., 2007; Ratnaparkhi et al., 2008). These factors/proteins may function as species-related co-regulators. *Ms*Lysozyme promoter showed similarly high activity in both S2 and Sf9 cells (Figure 1). We did not identify κB sites in *Ms*Lysozyme promoter by in silico analysis. Moreover, induced expression level of *Ms*Lysozyme mRNA by different bacterial components was always significantly lower than that of other AMP genes that are regulated by NF-κB factors (X-J Rao and X-Q Yu, unpublished data). Therefore, *Ms*Lysozyme is likely not regulated by NF-κB factors. It is also possible that regulation of AMP promoters may be tissue-specific, since S2 and Sf9 cells were used in this study, and S2 cells were hemocyte origin whereas Sf9 cells were developed from the ovary.

4.2 The κB, GATA and MPAE elements cooperatively induce gene transcription

MsMoricin κB-GATA element is necessary but not sufficient to activate *MsMoricin* promoter induced by *E. coli* peptidoglycan (Figures 2 and 3). This result indicates that other co-regulators are required to cooperate with NF-κB and GATA factors to activate transcription of *MsMoricin*. These co-regulators likely bind to the 140bp MPAE (MsMoricin Promoter Activating Element) region (Figures 2A and 3), which contains predicted binding sites for nuclear factors such as YY-1, Pit-1, Oct-1, and C/EBP. However, deletion of a predicted YY-1 (−201 to −192bp) and a Pit-1 (−185 to −176bp) site in MPAE did not have an effect on *MsMoricin* promoter activity (X-J Rao and X-Q Yu, unpublished data), suggesting that there may be novel co-regulator binding sites in the MPAE element.

4.3 The κB-GATA and MPAE elements activate D. melanogaster AMP gene promoters in S2 and SF9 cells differently

It has been reported that when *Drosophila* Toll and IMD pathways are stimulated simultaneously at a low level, there is a synergic effect on activation of *drosomycin* gene probably due to formation of Dorsal-Relish and/or Dif-Relish heterodimers that bind to the κB site2 (Tanji et al., 2007; Tanji et al., 2010). However, how Dorsal-Relish and/or Dif-Relish heterodimers synergistically activate *drosomycin* promoter is not well understood. We showed that the κB-GATA element from *MsMoricin* promoter could enhance activities of *Drosophila* AMP promoters (Figures 4–6) in S2 cells. We also showed that *drosomycin* promoter could be activated cooperatively by the endogenous κB site2 and the exogenous κB-GATA element (Figures 6B). Nonetheless, the same set of reporters consistently showed low activities in Sf9 cells (Figure S1), indicating that the κB-GATA element is not a species-related activating element.

MPAE specifically increased *drosomycin* promoter activity in Sf9 cells (Figure 8B), but not in S2 cells (Figure 8A), strongly suggesting that MPAE indeed contains lepidoptera-related co-regulator binding sites. Since the κB site1 is not activated by the IMD pathway (Tanji et al., 2007; Tanji et al., 2010), high activity of the $1rep^{(MPAE\text{-}\kappa B\text{-}GATA)}$-*Drs* reporter in S2 cells (Figure 7A) may result from synergic effect of the κB site2 and the κB-GATA element. But high activity of the $1rep^{(MPAE\text{-}\kappa B\text{-}GATA)}$-*Drs* reporter in Sf9 cells (Figure 7B) is due to both the MPAE and the κB-GATA elements. The κB site2 is activated by the IMD pathway (Tanji et al., 2007; Tanji et al., 2010), low activities of the MPAE and MPAE-κB replaced reporters ($2rep^{(MPAE)}$-*Drs* and the $2rep^{(MPAE\text{-}\kappa B)}$-*Drs*) (Figure 7C) suggest that an MPAE or an exogenous κB cannot substitute for the κB site2 to activate *drosomycin* in S2 cells.

However, a whole MPAE-κB-GATA element is a stronger element than the κB site2, since it could increase *drosomycin* activity to a significantly higher level in S2 cells (Figure 7A and C). Insertion of an MPAE alone before the site2 already caused a significant increase in promoter activity in Sf9 cells (2ins$^{(MPAE)}$-*Drs* reporter in Figure 7D). A whole MPAE-κB-GATA element increased activity in Sf9 cells to the highest level (2rep$^{(MPAE\text{-}\kappa B\text{-}GATA)}$-*Drs* reporter in Figure 7D). These results altogether indicate that MPAE contains lepidoptera-related regulators that can activate *drosomycin* promoter in Sf9 cells, and the *MsMoricin* κB-GATA is a stronger element than the endogenous κB site2 in activation of *drosomycin* promoter.

The κB-GATA element from *MsMoricin* increased activity of *diptericin* promoter in both S2 and Sf9 cells, and increased activity of *drosomycin* promoter only in S2 cells but not in Sf9 cells (Figures 4, 6 and S1). *Diptericin* is activated by the IMD pathway, while *drosomycin* is mainly activated by the Toll pathway. *MsMoricin* κB-GATA was also activated by the IMD pathway (Figure 3) as we used PG-K12 to stimulate cells. Thus, it is possible that an extra κB-GATA element and the endogenous κB sites in *diptericin* promoter act cooperatively to increase promoter activity in Sf9 cells.

4.4 Effect of the position, direction and consensus sequence of κB and GATA sites on AMP gene promoter activity

D. melanogaster Dorsal binds consensus sequence of GGG(A/T)(A/T)(T/A)(A/T/C)(C/A/T)(T/G/C); Relish binds consensus sequence of GGGA(A/T/C)N(C/T)(C/A)(C/T); Dif/Relish heterodimer binds consensus sequence of GGGA(A/T)TC(C/A)C (Busse et al., 2007; Senger et al., 2004). *Drosomycin* κB site1 (GGGTTTAACC) is consistent with Dorsal binding consensus; the κB site2 (GGGAACTACT) is consistent with Relish binding consensus; *MsMoricin* κB5 (GGGACTTTAC) is not completely consistent with any of the three consensus sequences. *MsMoricin* GATA site (CAGATAACGA) is consistent with *Drosophila* Serpent consensus sequence [(A/T/C)GATA(A/G)(C/T/G)] (Senger et al., 2004). Based on previous reports and our data, we propose that active RelN/RelN homodimer may bind to *MsMoricin* κB5 site and *drosomycin* κB site2, and a *Drosophila* GATA factor (Serpent, for example) may bind to *MsMoricin* GATA site to achieve maximal synergy in *drosomycin* promoter in S2 cells (Figure 9).

The position, direction and distance between the κB and GATA sites are also important for Rel-GATA synergy (Senger et al., 2004; Vardhanabhuti et al., 2007). The features of *MsMoricin* κB-GATA element used in our experiments are consistent with those reported previously (Figure 2A and Text S1) (Kadalayil et al., 1997; Senger et al., 2004). An *MsMoricin* GATA element alone could significantly increase *drosomycin* promoter activity in S2 cells when inserted after the endogenous κB site1 but did not show this effect when inserted after the κB site2 (Figure 8C). This may be due to special needs for the position, orientation and/or spacing of the inserted GATA element.

4.5 Potential applications of our finding

What we report here may have broad applications in transgenic engineering to increase antibacterial activities in different organisms. A κB-GATA element may be inserted into a promoter to drive expression of a transgene only after induction by bacteria, and a species-specific element, if identified, may be inserted into a promoter to drive expression of an exogenous gene in an organism. Plants do not have NF-κB factors, but they do have GATA factors and AP-1 like WRKY factors (Reyes et al., 2004; Ronald and Beutler, 2010). AP-1 and GATA-2 cooperatively regulate expression of Endothelin-1, a vasoactive peptide from endothelial cells (Kawana et al., 1995). Thus, WRKY-GATA synergy might exist in plants too, although there have been no reports so far. More research is needed to further identify

unknown co-regulators in the transcription complex as this will shed light on molecular mechanisms of immune gene regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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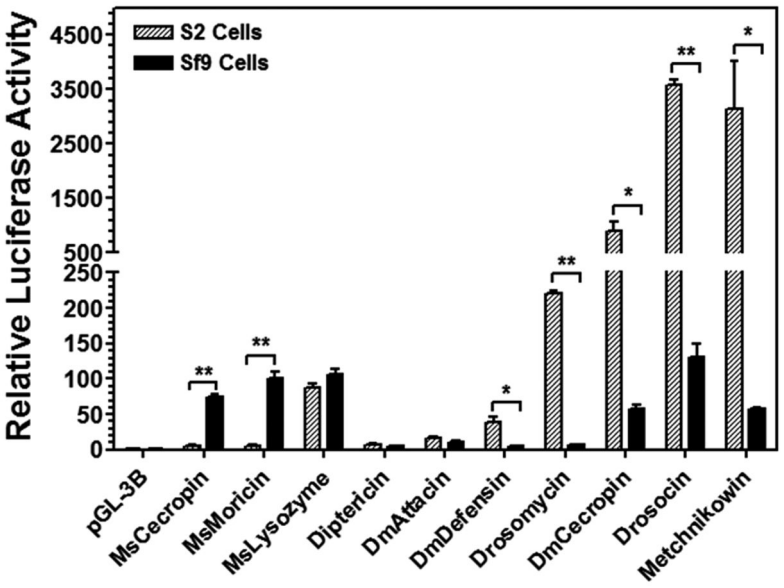

Figure 1. Species-specific regulation of AMP gene promoters in S2 and Sf9 cells

S2 and Sf9 cells were transiently cotransfected with different reporter vectors using pRL-TK as an internal control. PG-K12 (peptidoglycan from *E. coli* K12 strain, 10 μg/ml final concentration) was used to stimulate cells for 48 h before luciferase activities were measured. Relative luciferase activity was normalized to the pGL-3B control group (arbitrarily set as 1). Bars represent the mean of three individual measurements ± S.E.M. Significance of difference between S2 and Sf9 cells in each group was determined by an unpaired t-test (* $p<0.05$; ** $p<0.001$).

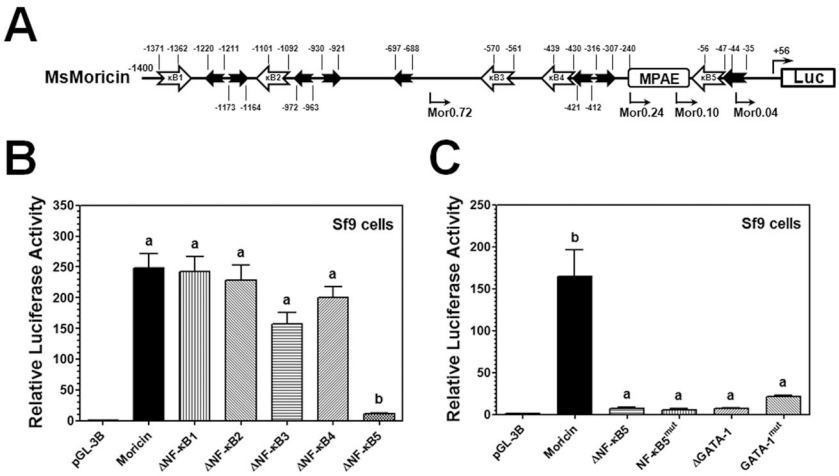

Figure 2. Identification of an active κB-GATA element in *MsMoricin* promoter

MsMoricin promoter was cloned by genome walking. Luciferase activities were measured in PG-K12 stimulated Sf9 cells. (A) Schematic representation of *MsMoricin* promoter. In silico analysis showed five predicted κB sites (open arrowed boxes) and eight GATA binding sites (filled arrowed boxes) with one GATA-1 site adjacent to the κB5. MPAE: *MsMoricin* Promoter Activating Element. (B) The κB5 site is required for promoter activity. Each of the five κB sites was deleted seperately, and only deletion of the κB5 caused a complete loss of promoter activity. (C) Both the κB5 and the adjacent GATA-1 sites are required for promoter activity. The κB5 or GATA-1 site was deleted or mutated separately. Deletion or mutation of either the κB5 or the adjacent GATA-1 site caused a significantly loss in promoter activity. Bars represent the mean of three individual measurements ± S.E.M. Identical letters are not significant difference between groups ($p>0.05$), while different letters indicate significant difference between groups ($p<0.05$) determined by one way ANOVA followed by a Tukey's multiple comparison test.

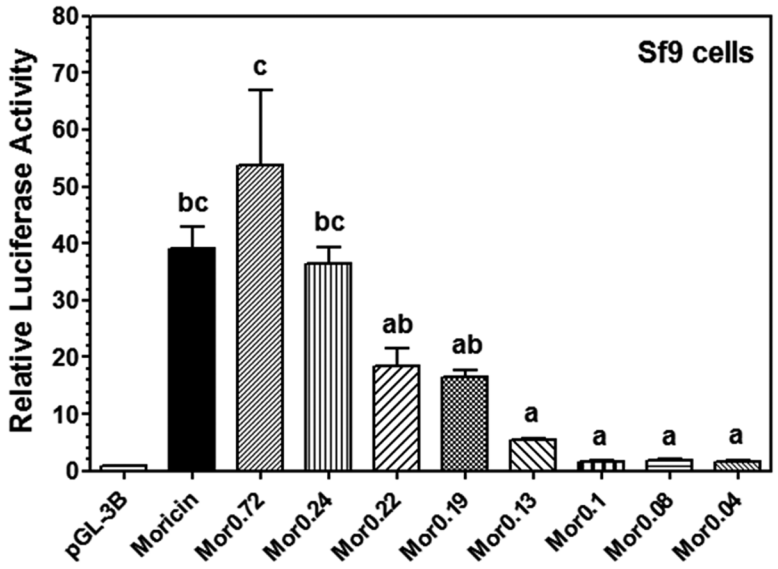

Figure 3. Identification of an *MsMoricin* Promoter Activating Element (MPAE)

MsMoricin promoter deletion mutants were constructed by shortening the 5′ end. Luciferase activities of these promoters were measured in Sf9 cells as described in Figure 2 (Refer to Figure 2A for the schematic representation).

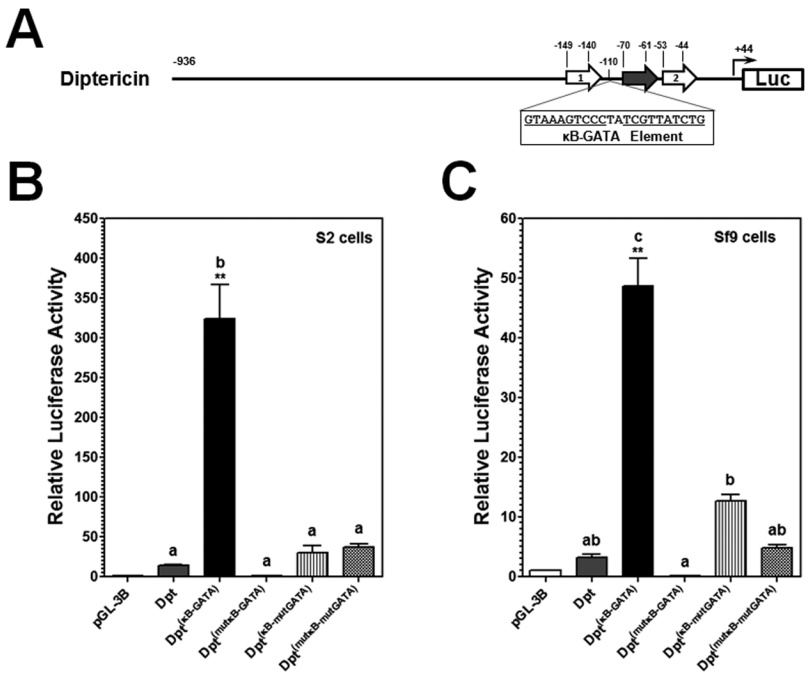

Figure 4. *MsMoricin* κB5-GATA element can increase *Drosophila diptericin* promoter activity in S2 and Sf9 cells

(A) Schematic representation of *diptericin* promoter. Open arrowed boxes indicate κB sites and filled arrowed box indicates GATA-1 site in *diptericin* promoter (Kappler et al., 1993; Reichhart et al., 1992; Senger et al., 2004). An *MsMoricin* κB5-GATA element or a mutant κB5-GATA element (mutation in either the κB5 or the GATA-1 site, or both sites) was inserted into *diptericin* promoter at the −110 position (represented by $Dpt^{(\kappa B\text{-}GATA)}$, $Dpt^{(mut\kappa B\text{-}GATA)}$, $Dpt^{(\kappa B\text{-}mutGATA)}$, or $Dpt^{(mut\kappa B\text{-}mtGATA)}$). Activities of these *diptericin* promoters were determined in S2 cells (B) or Sf9 cells (C) as described in Figures 1 and 2.

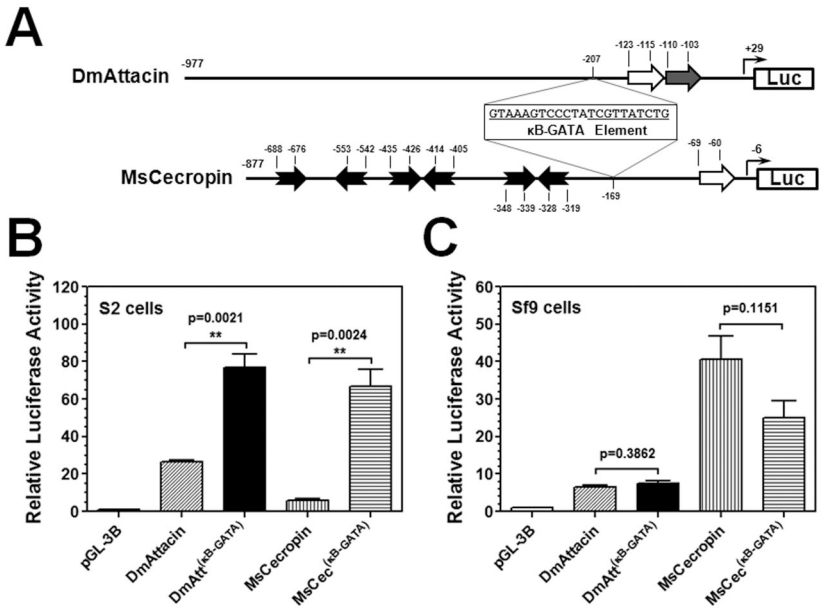

Figure 5. The κB5-GATA element can increase *DmAttacin* A and *MsCecropin* promoter activities in S2 cells

(A) Schematic representation of *DmAttacin* A and *MsCecropin* promoters. An NF-κB and a GATA sites in *DmAttacin* A promoter have been identified (Senger et al., 2004; Tanji et al., 2007), and an NF-κB site and six GATA sites in *MsCecropin* promoter were predicted. An *MsMoricin* κB5-GATA element was inserted into *DmAttacin* A promoter at the −207 position and into *MsCecropin* promoter at the −169 position. Activities of these promoters were determined in S2 cells (B) or Sf9 cells (C) as described in Figures 1 and 2.

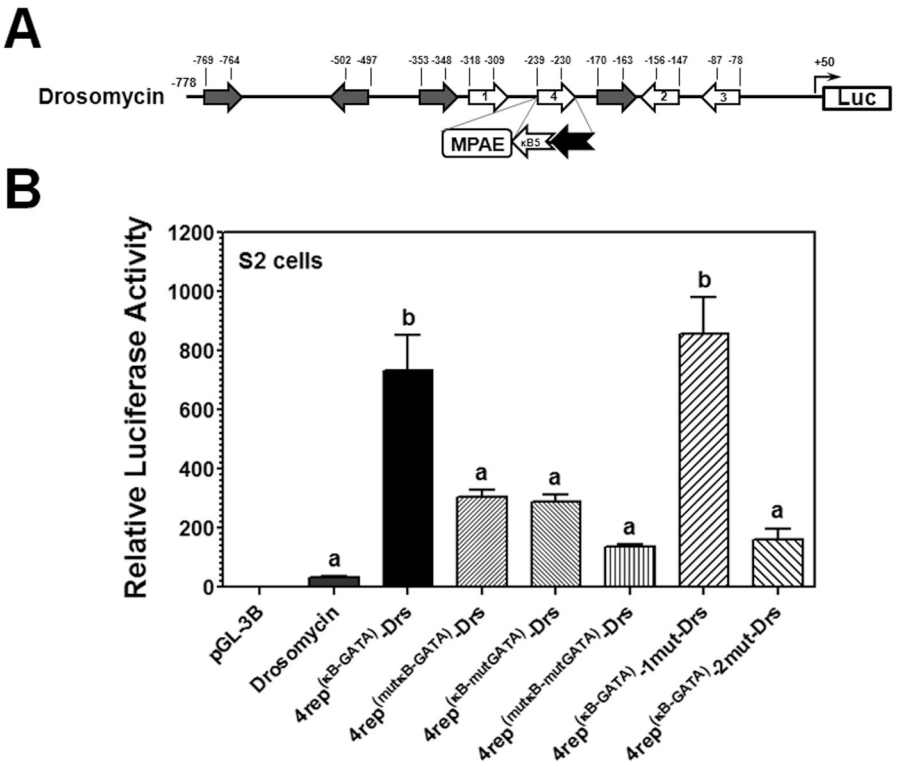

Figure 6. The κB5 and GATA elements act cooperatively with *drosomycin* κB site2 to increase *drosomycin* promoter activity

(A) Schematic representation of *drosomycin* promoter. The NF-κB site1, site2 and site3 have been experimentally confirmed (Tanji et al., 2007; Tanji et al., 2010), and four GATA binding sites have been identified (Senger et al., 2004). A κB site4 was predicted. The predicted κB site4 of *drosomycin* promoter was deleted or mutated (represented by 4del-*Drs* or 4mut-*Drs*), or replaced with an *MsMoricin* κB5-GATA element or a mutant κB5-GATA element (mutation in either the κB5 or the GATA-1 site, or both sites) (represented by $4rep^{(\kappa B\text{-}GATA)}$-*Drs*, $4rep^{(mut\kappa B\text{-}GATA)}$-*Drs*, $4rep^{(\kappa B\text{-}mutGATA)}$-*Drs*, or $4rep^{(mut\kappa B\text{-}mtGATA)}$-*Drs*). The κB site1 or site2 in the κB5-GATA replaced promoter was also mutated (represented by $4rep^{(\kappa B\text{-}GATA)}$-1mut-*Drs* or $4rep^{(\kappa B\text{-}GATA)}$-2mut-*Drs*). Activities of these promoters were determined in S2 cells (B) or in Sf9 cells (Figure S1, panels A and B) as described in Figures 1 and 2.

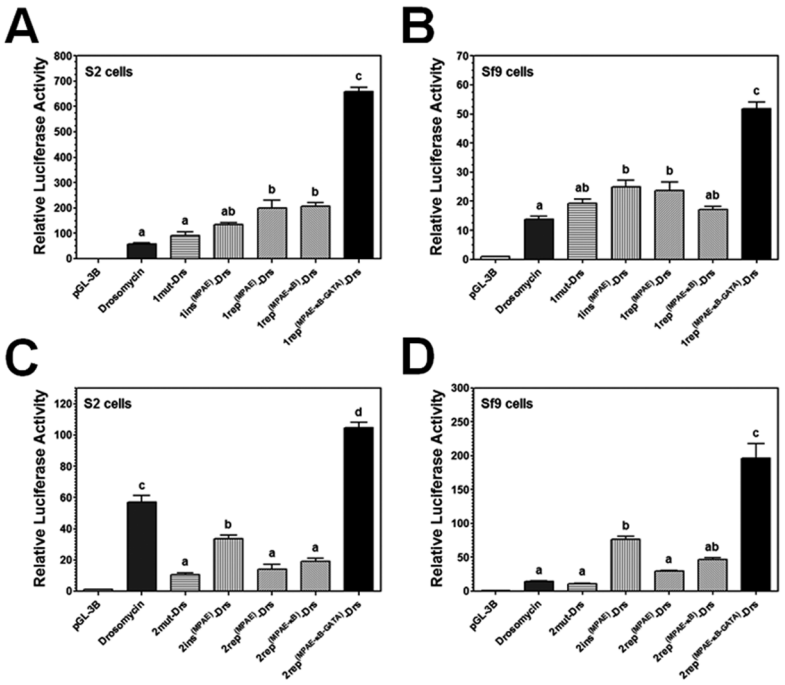

Figure 7. MPAE can specifically increase *drosomycin* promoter activity in Sf9 cells

The κB site1 or site2 in *drosomycin* promoter was mutated, or replaced with an MPAE, MPAE-κB5 or MPAE-κB5-GATA (represented by 1mut-*Drs*, 1rep$^{(MPAE)}$-*Drs*, 1rep$^{(MPAE-κB)}$-*Drs*, or 1rep$^{(MPAE-κB-GATA)}$-*Drs* for the site1, and the same for the site2). An MPAE was also inserted just before the site 1 or site2 (represented by 1ins$^{(MPAE)}$-*Drs* or 2ins$^{(MPAE)}$-*Drs*). Activities of these promoters were determined in S2 cells (A and C) or Sf9 cells (B and D) as described in Figures 1 and 2.

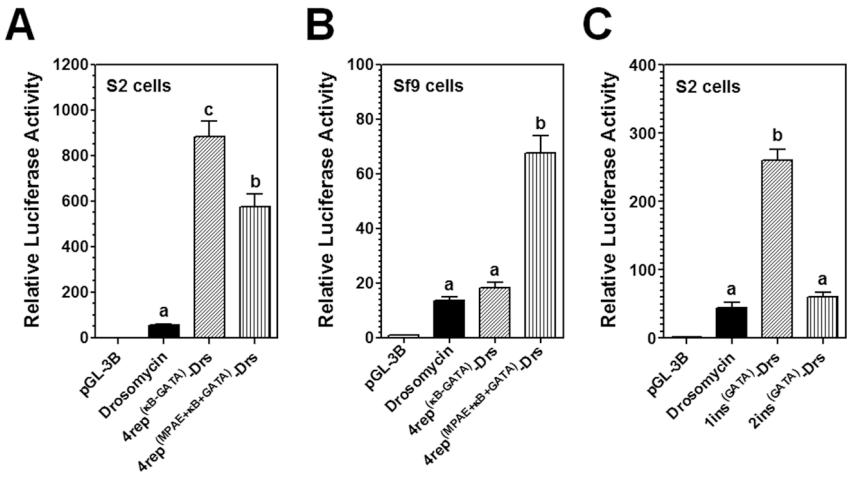

Figure 8. Effects of the GATA, κB-GATA and MPAE-κB-GATA elements on *drosomycin* promoter activity

MsMoricin GATA-1 element was inserted immediately after the κB site1 or site2 of *drosomycin* promoter (representing by 1ins$^{(GATA)}$-*Drs* or 2ins$^{(GATA)}$-*Drs*), or the predicted κB site4 of *drosomycin* promoter was replaced with an *MsMoricin* κB5-GATA or an MPAE-κB5-GATA element (represented by 4rep$^{(κB\text{-}GATA)}$-*Drs* or 4rep$^{(MPAE\text{-}κB\text{-}GATA)}$-*Drs*). Activities of these promoters were determined in S2 cells (A and C) or Sf9 cells (B and Figure S1, panel C) as described in Figures 1 and 2.

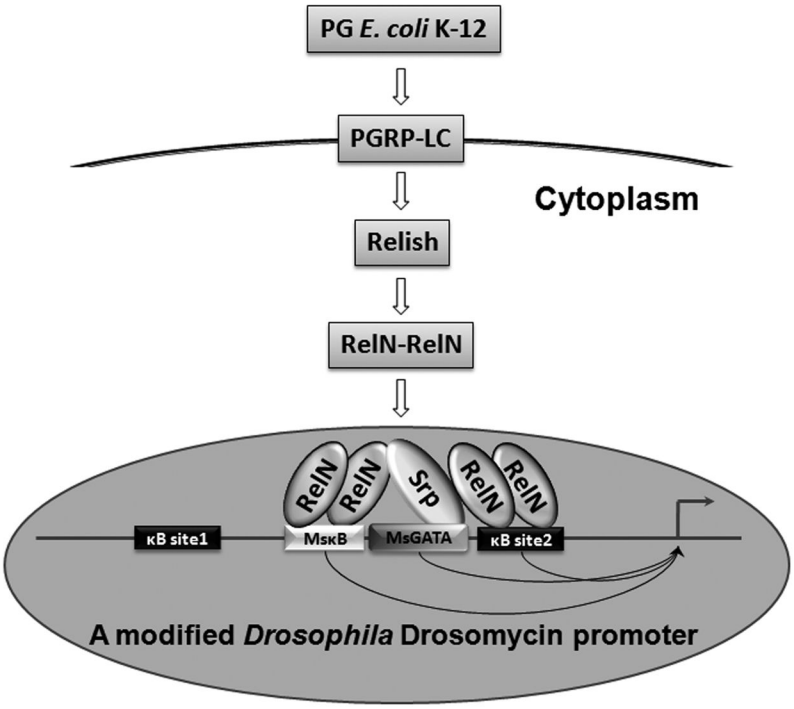

Figure 9. A model for synergistic effect of an exogenous κB and GATA elements with the endogenous κB element on *drosomycin* promoter activity

PG-K12 activates the Imd pathway, leading to formation of active RelN/RelN homodimers that bind to the exogenous and endogenous κB sites. A *Drosophila* GATA factor (Serpent) binds to the exogenous GATA-1 site and probably brings the two κB sites together to facilitate formation of a transcription complex.